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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Group:	1638
Confirmation No.:	9526
Application No.:	09/486,904
Invention:	SELECTIVE EXPRESSION OF GENES IN PLANTS
Applicant:	Snyder, et al.
Filed:	March 3, 2000
Attorney Docket:	3220-66107

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March 14, 2011

AMENDED APPEAL BRIEF

Mail Stop Appeal Brief - Patents
Director of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants electronically submit this Amended Appeal Brief in response to the Notification of Non-Compliant Appeal Brief mailed December 14, 2010. The one-month time period for filing this Amended Appeal Brief was set by the Office to January 14, 2011. Appellants request a two-month extension of time to extend the deadline for filing this Amended Appeal Brief from January 14, 2011, to March 14, 2011. Appellants hereby authorize the Director to charge the fee for two months extension of time ($490.00), as well as any additional fees, and/or credit any overpayments, to Deposit Account No. 10-0435, with reference to our file number 3220-66107.

REAL PARTY IN INTEREST

The real party in interest is Purdue Research Foundation, the assignee, pursuant to an assignment by the inventors, recorded in the U.S. Patent and Trademark Office at Reel 9771, Frame 0658 on February 19, 1999, for the rights in the parent PCT application, PCT/US98/18416.

RELATED APPEALS AND INTERFERENCES

There are no other pending appeals or interferences related to the present appeal.

STATUS OF CLAIMS

Claims 9 and 20-25 are pending in the captioned application. Claims 1-8, 15-19 have been cancelled. Claims 10-14 have been withdrawn as being drawn to a non-elected group. Each of claims 9 and 20-25 were rejected in the final Office Action dated September 23, 2003. Claims 9 and 20-25 were appealed in the Notice of Appeal dated December 19, 2003.

STATUS OF AMENDMENTS

Appellants have made no amendments to the claims subsequent to the Final Office Action.

SUMMARY OF CLAIMED SUBJECT MATTER

Independent Claim 9:

Independent claim 9 recites a method for producing a compound in a transgenic plant. The method comprises the steps of producing a fertile transgenic plant by introducing into plant cells a DNA construct comprising a promoter (2), a blocking sequence (Fig. 1A (a)), and a coding sequence of a structural gene coding for a compound that is detrimental to the plant and is commercially valuable, the blocking sequence being flanked by a pair of directly repeated site-specific recombination sequences (4) and wherein the structural gene is operably linked to the promoter only after the removal of the blocking sequence, and culturing the plant cells to produce the fertile transgenic plant; pollinating the transgenic plant to produce

transgenic plants that are homozygous for the DNA construct; crossing the transgenic plant homozygous for the DNA construct with a plant having a DNA sequence comprising a coding region encoding a site-specific recombinase (Fig. 1D, (12)) that recognizes the site-specific recombination sequences (4) to produce an F1 plant or seed; expressing the site-specific recombinase in the F1 plant or seed; expressing the compound; and extracting the compound in economical quantities.

The method of claim 9 is further described in the passages of the specification on page 5, line 28 through page 6, line 16.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Appellants present to following grounds of rejection for the Board to review:

(I) the rejection of claims 9 and 20-25 alleging indefiniteness under 35 U.S.C. § 112, second paragraph, for failing to particularly point out and distinctly claim the invention;

(II) the rejection of claims 9 and 20-25 under 35 U.S.C. § 112, first paragraph, alleging failure enable one to make and use the invention commensurate with the scope of the claims; and

(III) the rejection of claims 9 and 20-25 alleging obviousness under 35 U.S.C. § 103(a) over Kilby, NJ (1995), Plant Journal 8: 637-652, in view of Odell, et al., U.S. Patent No. 5,658,772, and Kilby, NJ (1993), Trends in Genetics 9: 413-421.

ARGUMENT

I. APPELLANTS URGE THE BOARD TO REVERSE THE FIRST GROUND OF REJECTION.

Appellants will argue the claims within the first ground of rejection in the following group:

Group A – claims 9 and 20-25.

A. CLAIMS 9 AND 20-25 ARE NOT INDEFINITE

Claims 9 and 20-25 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. Initially, the Examiner finds that the term "gene" of claim 9 is unclear. According to the Examiner, the term "gene" implies a DNA sequence that exists in nature and includes coding and noncoding regions, as well as regulatory sequences associated with expression. Applicants respectfully submit that the term "gene" may be used to in other ways, for example the portion of the DNA that is transcribed into RNA. In a previous effort to advance prosecution and to clarify the claim language, the applicants replaced the term "structural gene" with "coding sequence of a structural gene." Applicants believe that this language is now clear and does not require further amendment. However, if all other issues are resolved, applicants would not object to the Examiner's suggested language in lines 5 and 7 of claim 9.

The Examiner has also rejected claim 9 because the Examiner finds that the term "economical quantities" is unclear. Applicants have previously submitted that the term "economical quantities" refers to a compound that is produced in sufficient quantities that the value of the extracted compound exceeds the costs associated with standard production and extraction methods. The Examiner now contends that the applicants have failed to define "standard production costs." Applicants submit that while standard production costs would vary from crop to crop, and for extraction of various compounds, and such costs would be readily determinable by a production facility. Applicants respectfully submit that the exact parameters of production costs need not be specified to render this claim definite.

Reconsideration of the rejection of claims 9 and 20-25 under 35 U.S.C. § 112, second paragraph, leading to reversal of the Examiner's rejection and passage of the application to issuance is respectfully requested.

II. APPELLANTS URGE THE BOARD TO REVERSE THE SECOND GROUND OF REJECTION.

Appellants will argue the claims within the second ground of rejection in the following group:

Group A – claims 9 and 20-25.

A. CLAIMS 9 AND 20-25 ARE ENABLED

Claims 9 and 20-25 stand rejected under 35 U.S.C. § 112, first paragraph. According to the Examiner, the specification, while being enabling for a method of producing a compound that is not detrimental to the plant, is not enabling for a compound that is detrimental to the plant. According to the Examiner, the state of the art is that one skilled in the art can readily make DNA constructs containing a structural gene encoding a compound, transform these into plants, and express them with a reasonable expectation of success. However, according to the Examiner, expressing a compound detrimental to the plant, such as barnase, is more unpredictable, and that expression of a gene encoding such a detrimental compound must be regulated in order to avoid killing all cells expressing the compound. Further, according to the Examiner, recombinase mediated excision of appropriately flanked DNA sequences is variable and yields chimeric phenotypes having both recombined and unrecombined DNA. The Examiner has previously cited Gidoni, D, et al. (2001) Euphytica 121:145-156, for the proposition that embryonal recombination and germline inheritance of recombined tobacco loci show variable recombination efficiencies, and Vergunst (1998) Nuc. Acids. Res. 26: 2729-2734, for the unpredictability of using the recombinase system as evidenced by instability of recombinants and phenotypic "escapes." Thus, the Examiner concludes that, without further guidance, it is unpredictable that one skilled in the art would be able to express in a plant a structural gene encoding a compound that is detrimental to the plant, as applicants have provided no guidance on how to eliminate predictably inoperable embodiments.

First of all, the specification of the above-captioned application provides ample proof-of-concept examples. While these examples, as discussed on pages 17-31 of the specification, use compounds that are not detrimental, the examples clearly demonstrate that the

claimed methods can be used to control expression of a compound for the purposes of extracting that compound, and it is within the ordinary skill in the art to substitute one coding sequence for another. Yet, the Examiner summarily finds that "expressing structural genes encoding a compound detrimental to the plant, such as barnase, is more unpredictable." However, the Examiner provides no basis for this assertion. Applicants question why it would be more unpredictable to express a detrimental compound in the claimed recombination methods, when such methods provide the regulation of expression needed with a compound such as barnase. Even if it is difficult to control barnase expression, merely because expression of a single very lethal compound, barnase, may be difficult to control, such difficulty does remove expression of the group of detrimental compounds from a person skilled in the art. Additionally, even if a transgenic line were "leaky" and some of the detrimental compound were expressed too early in some plants, such would reduce the yield, and perhaps even kill a percentage of the transgenic plants, but this would not render the invention inoperative. The Examiner simply has not substantiated an enablement rejection based on the difference in the predictability of expression of detrimental verses non-detrimental compounds.

Furthermore, applicants respectfully submit that recent work has demonstrated that site-specific recombinases can be nearly 100% efficient. Attached to the response of May 27, 2003 (resubmitted June 16, 2003) as Exhibit A is Zuo et al. (2001) Nature Biotechnology 19: 157-161. In Zuo, all 19 of the Arabidopsis lines created using a Cre/Lox mediated excision system underwent excision. Luo et al. (2000) The Plant Journal 23(3): 423-430, attached to the response of May 27, 2003 (resubmitted June 16, 2003) as Exhibit B, shows similar success with FLP/FRT, the site-specific recombinase system of claim 23. Instances where the efficiencies of the recombinases were found to be low can be attributed to weak promoters, or, as discussed by Luo, may be due to position effects (Luo at p. 427). Genes may be silenced due to these position effects, thus negating their expression. These deleterious effects may be overcome by selecting the transgenic lines whose transgenes are not silenced. Such selection is within the ordinary skill in the art.

Appellants acknowledge that the Examiner has noted that the Zuo and Luo references were not previously submitted in an Information Disclosure Statement. However,

these references are post-filing date references and are not prior art. These references are being submitted only to refute the Examiner's claims of lack of predictability, as evidenced by the Examiner's own post-filing date references. Accordingly, Appellants do not believe that an Information Disclosure Statement is required. If an Information Disclosure Statement is required, Appellants would be happy to submit one.

The Examiner also rejects the Zuo and Luo references because these references do not contain the exact method as the instant case. In particular, the Examiner points out that different promoters are used and the recombinase sequences remove different blocking sequences. However, the presently appealed claims do not require a specific promoter and blocking sequence. Furthermore, the Appellants' references are at least as or more similar to the disclosed embodiments than several of the references cited by the Examiner. For example, Luo use FRT/FLP, as in the examples provided in the present specification, whereas the Vergunst reference cited by the Examiner uses the same promoter that the Examiner criticizes in Zuo, 35S CaMV, and uses the Cre/Lox recombination system, rather than FRT/FLP. Thus, the Zuo reference is closer to the disclosed embodiments than that of the Vergunst reference, and the group of references cited by the appellants are at least, if not more, applicable than the group of references cited by the Examiner.

In sum, Appellants respectfully submit that the Examiner made improper conclusory statements regarding unpredictability in expressing structural genes encoding compounds in plants and the Examiner improperly relied on post-filing date references to find lack of enablement, while improperly dismissing Appellants' post-filing date references of equal or greater relevance. Appellants respectfully submit that the art is sufficiently predictable to enable one of ordinary skill in the art to make and use the invention. Accordingly, reconsideration of the rejection of claims 9 and 20-25 under 35 U.S.C. § 112, first paragraph, leading to reversal of the Examiner's rejection and passage of the application to issuance is respectfully requested.

III. APPELLANTS URGE THE BOARD TO REVERSE THE THIRD GROUND OF REJECTION.

Appellants will argue the claims within the third ground of rejection in the following group:

Group A – claims 9 and 20-25.

A. CLAIMS 9 AND 20-25 ARE NOT OBVIOUS OVER KILBY (1995) IN VIEW OF ODELL ('772 PATENT) AND KILBY (1993)

Claims 9 and 20-25 stand rejected under 35 U.S.C. § 103(a) as being obvious over Kilby (1995), Plant Journal 8: 637-652, in view of Odell, U.S. Patent No. 5,658,772, and Kilby (1993), Trends in Genetics 9: 413-421. The Examiner finds that the term "extracting the compound in economical quantities" is given no patentable weight due to lack of clarity of this claim term. Accordingly, the Examiner reiterates a prior rejection from an Office Action mailed January 31, 2002 stating on pages 6 to 7:

> Kilby (1995) teaches introducing into plant cells a DNA construct comprising a promoter, a blocking sequence, and a structural gene, where the blocking sequence is flanked by a pair of directly repeated site-specific recombination sequences wherein the structural gene is operably linked to the promoter only after the removal of the blocking sequence (Figure 2, page 639). Kilby does not teach biological detrimental compounds. Odell et al teach detrimental compounds (column 14, lines 15, 25 and 26). The toxicity of the detrimental biological compound itself provides motivation for blocking expression until the desired time. Kilby (1993) (pg 420) suggest the strategy of gene activation only after removal of a blocking sequence as being particularly useful for expressing potentially harmful genes. It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to use the strategy of expressing a biologically detrimental compound only after removal of a blocking sequence. One skilled in the art would have been motivated to generate

> the claimed invention with a reasonable expectation of success. Odell further teaches the site-specific recombination system of Cre recombinase and Lox site specific recombination sequences. It is know [sic] in the art that the Cre/lox and the FLP/frt site-specific recombination systems (composed of the recombinase enzyme –either Cre of [sic] FLP, and their cognate recombination target sites – either lox or frt respectively) are functional equivalents (see Kilby et al, 1993, (V)). These are known prior art systems for addressing the same problem. Accordingly, the use of Cre/lox or the FLP/frt recombination system is a matter of design choice well within the means of one of ordinary skill without any surprising or unexpected results.

As discussed above, Appellants respectfully submit that the term "economical quantities" refers to a compound that is produced in sufficient quantities that the value of the extracted compound exceeds the costs associated with standard production and extraction methods. The Examiner has maintained this rejection because the Examiner finds that this definition "does not teach what 'standard production costs are.'" Appellants submit that standard production costs would vary from crop to crop, and for extraction of various compounds. While Appellants do not define standard production costs, such costs should be easily and readily determinable.

Furthermore, even assuming, *arguendo*, that it "would have been *prima facie* obvious and well within the means of one of ordinary skill in the art at the time the invention was made to use the strategy of expressing a biologically detrimental compound only after removal of a blocking sequence," Appellants respectfully submit that none of the Kilby or Odell references, alone or in combination, suggest the use of the claimed structures for the expression and extraction of the detrimental compound, and the references certainly do not teach or suggest extraction of economical quantities of the compound. Instead, Odell teaches the use of a construct to produce barnase for *in situ* disruption of the seed development. Odell is silent on the quantity of barnase produced. Indeed, Odell does not even indicate that an

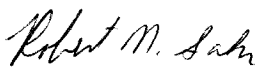
extractable amount of barnase would be produced, and certainly not an economical quantity. While Odell teaches extraction of DNA and RNA for analysis purposes, this is different and clearly distinct from extraction of the resultant barnase protein, which may or may not be present and stable in extractable quantities. Further, because the barnase is used for *in situ* disruption of the seed development to produce seedless watermelon, extraction of the barnase would be counter to the disclosed use of this detrimental compound. Also, that Kilby (1993) suggests the use of a construct for the study of harmful mutations in no way teaches or suggests that a mutation gene product could be expressed and extracted in economic quantities. Kilby (1995) is completely silent regarding detrimental compounds and, thus, does not suggest expression and extraction of such compounds in quantities sufficient for commercial production. Odell, alone or in combination with the Kilby references, simply fails to teach or suggest production of a detrimental compound and extraction of that same compound.

Reconsideration of the rejection of claims 9 and 20-25 under 35 U.S.C. § 103(a) leading to reversal of the Examiner's rejection and passage of the application to issuance is respectfully requested.

IV. CONCLUSION

In view of the arguments presented above, Appellants submit that the three grounds of rejection are erroneous. Appellants urge the Board to reverse the rejection of the pending claims and respectfully request action to that end.

Respectfully submitted,



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CLAIMS APPENDIX

Appellants submit the following list of claims involved in the appeal in accordance with 37 C.F.R. § 41.37(c)(1)(viii):

9. A method for producing a compound, said method comprising the steps of

producing a fertile transgenic plant by introducing into plant cells a DNA construct comprising a promoter, a blocking sequence, and a coding sequence of a structural gene coding for a compound that is detrimental to the plant and is commercially valuable, said blocking sequence being flanked by a pair of directly repeated site-specific recombination sequences and wherein the structural gene is operably linked to the promoter only after the removal of said blocking sequence, and culturing the plant cells to produce the fertile transgenic plant;

pollinating said transgenic plant to produce transgenic plants that are homozygous for the DNA construct;

crossing said transgenic plant homozygous for the DNA construct with a plant having a DNA sequence comprising a coding region encoding a site-specific recombinase that recognizes said site-specific recombination sequences to produce an F1 plant or seed;

expressing the site-specific recombinase in the F1 plant or seed;

expressing the compound; and

extracting the compound in economical quantities.

20. The method of claim 9 wherein the step of crossing said homozygous transgenic plant with a plant having a DNA sequence comprising a coding region encoding a site-specific recombinase produces an F1 plant or seed that expresses the biologically detrimental compound.

21. The method of claim 20, wherein the extracting step comprises extracting the compound from the plant or seed.

22. The method of claim 9 wherein the promoter is a constitutive promoter.

23. The method of claim 9 wherein the pair of directly repeated site-specific recombination sequences are FRT recombination sequences, and the coding region encoding the site-specific recombinase encodes the FLP recombinase and is operably linked to a constitutive promoter.

24. The method of claim 9 wherein the step of pollinating said transgenic plant to produce plants that are homozygous for the DNA construct comprises self-pollination.

25. The method of claim 9 wherein the promoter of the DNA construct is a leaf-specific promoter and the extracting step includes extracting the compound from leaves.

EVIDENCE APPENDIX

1. Luo et al. (2000) *The Plant Journal* 23(3): 423-430

2. Zuo et al. (2001) *Nature Biotechnology* 19: 157-161

The Plant Journal (2000) **23**(3), 423–430

TECHNICAL ADVANCE

FLP-mediated recombination for use in hybrid plant production

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Summary

We have studied the feasibility in *Arabidopsis* of using a site-specific recombination system FLP/*FRT*, from the 2μm plasmid of yeast, for making plant hybrids. Initially, *Arabidopsis* plants expressing the FLP site-specific recombinase were crossed with plants transformed with a vector containing kanamycin-resistance gene (*npt*) flanked by *FRT* sites, which also served to separate the CaMV35S promoter from a promoterless *gusA*. Hybrid progeny were tested for excision of the *npt* gene and the positioning of 35S promoter proximal to *gusA*. GUS activity was observed in the progeny of all crosses, but not in the progeny derived from the self-pollinated homozygous parents. We then induced male sterility in *Arabidopsis* plants using the antisense expression of a pollen- and tapetum-specific gene, *bcp1*, flanked by *FRT* sites. Upon cross-pollination of flowers on the same male-sterile plants with pollen from FLP-containing plants, viable seeds were produced and the progeny hybrid plants developed normally. Molecular analyses revealed that the antisense expression cassette of *bcp1* had been excised in these plants. These results show for the first time that a site-specific recombinase can be used to restore fertility in male-sterile plants, providing an alternative method for the production of hybrid seeds and plants.

Keywords: FLP/*FRT*, site-specific recombination, male-sterility, hybrid, *Arabidopsis*, antisense.

Introduction

Hybrids of most crop plants yield 10–30% more than pure inbred lines (Mayo, 1980). The most successful crops utilizing hybrids have been corn and rice. In both crops, cytoplasmic male-sterile (CMS) plants have been successfully used on a large scale in hybrid seed production. However, the use of a unique type of cytoplasm has drawbacks because of the increased vulnerability of the plant to insects and pathogens (Levings, 1990). Therefore alternative methods for producing hybrid plants are highly desirable. In corn, detasseling (removal of the male part of line A) and then pollination by line B for hybrid production is an alternative, but detasseling is costly. Neither detasseling nor CMS systems are available for many economically important crops such as wheat, soybean, canola, or barley, which are still bred and grown as inbred varieties.

An alternative to CMS, and detasseling in the case of corn, is the development of male sterility by the selective ablation of tapetal cells, which are essential for the successful development of pollen (Mariani *et al.*, 1990; Moffatt and Somerville, 1988; Tsuchiya *et al.*, 1995; Xu *et al.*, 1995a). Selective ablation of tapetal cells by cell-specific expression of cytotoxic molecules (Mariani *et al.*, 1990) or an antisense gene (Xu *et al.*, 1995a) blocks pollen development, resulting in male sterility. By blocking the expressed cytotoxic molecules through crossing to a plant that expresses an inhibitor of the cytotoxin, Mariani *et al.* (1990, 1992) were able to restore fertility and thus produce hybrid plants. However, methods to restore the fertility for the antisense-caused male sterility have not been developed. In this instance, as well as in the cytotoxic

strategy, site-specific recombinases may provide a versatile method to restore fertility.

Site-specific recombinases are enzymes that recognize specific DNA sequences, and in the presence of two such recombination sites they catalyze the recombination of DNA strands (Ow and Medberry, 1995). In these site-specific recombination systems, recombinases can catalyze excision or inversion of a DNA fragment according to the orientation of their specific target sites. Recombination between directly oriented sites leads to excision of the DNA between them, whereas recombination between inverted target sites causes inversion of the DNA between them. Some site-specific recombination systems do not require additional factors for their function and are capable of functioning accurately and efficiently in various heterologous organisms. For example, FLP/*FRT* from the 2 μm plasmid of *Saccharomyces cerevisiae* (Broach *et al.*, 1982) and Cre/*lox* from *E. coli* phage P1 (Austin *et al.*, 1981) have been shown to catalyze DNA recombination efficiently in plant cells (Bar *et al.*, 1996; Bayley *et al.*, 1992; Dale and Ow, 1991; Kilby *et al.*, 1995; Lloyd and Davis, 1994; Lyznik *et al.*, 1993; Lyznik *et al.*, 1996; Odell *et al.*, 1990; Russel *et al.*, 1992; Sonti *et al.*, 1995; Srivastava *et al.*, 1999).

Here we have studied the feasibility of using a site-specific recombinase, specifically FLP in *Arabidopsis* plants, for making plant hybrids. Using this system in transgenic male-sterile plants we have successfully eliminated the male sterility-causing elements integrated into the host genome by crossing with pollen from a plant expressing the FLP recombinase, yielding hybrid seeds.

In the hybrid/recombinase system described here, male sterility can be induced by any pollen- or tapetum cell-specific gene whose malfunction causes male sterility. This male sterility-causing gene would ultimately be excised from the host genome. This is unlike the *barnase/barstar* hybrid plant system (Mariani *et al.*, 1992), in which the barnase protein (an RNase) causes male sterility, and fertility is restored by complexing to barstar, an inhibitor of the RNase, blocking the barnase activity. In the present study, male sterility was induced in *Arabidopsis* using the antisense of the *bcp1* gene which had previously been shown by Xu *et al.* (1995b) to induce male sterility in *Arabidopsis*. We were then able to use FLP to excise the antisense *bcp1* gene to restore fertility. Thus the recombination system offers much more flexibility in designing a sterility–fertility restoration system for hybrid seed production.

Results

In planta *test of efficacy of FLP-mediated recombination*

To test the efficacy of FLP recombination activity when expressed in plants, we first obtained two transgenic lines of *Arabidopsis thaliana* ecotype Columbia using FLP-containing construct pJFLO and the recombination–reporter construct, pFFG, respectively (Figure 1). In the pFFG construct, the presence of a 1.31 kb *npt* fragment flanked by directly oriented *FRT* sites between the CaMV 35S promoter–omega enhancer and the *gusA* coding region prevented *gusA* transcription. Excision of the blocking sequence (*npt* gene) by FLP will bring together the 35S promoter and the downstream *gusA* reporter gene, giving rise to *gusA* expression.

After *Agrobacterium tumefaciens*-mediated transformation, transgenic plants were selected for their kanamycin resistance and the presence of the gene was confirmed by Southern analysis (data not shown). Homozygous transgenic lines with one single integrated transgene were obtained by selfing and monitoring for segregation for kanamycin resistance. Two transgenic *Arabidopsis* lines transformed with FLP-containing construct pJFLO (P7) and the recombination–reporter construct, pFFG, respectively (Figure 1a,b) were chosen for subsequent experiments.

After cross-pollination between the FLP-expressing plant (P7) and *FRT*-containing plants, hybrid seedlings were harvested and stained for GUS activity. In total, flowers of 24 *FRT*-containing plants were hand-pollinated with pollen from the FLP-expressing plant (P7). Fifty randomly chosen hybrid seedlings from each crossing event were tested for GUS expression. All the progeny seedlings tested stained blue and exhibited a more-or-less uniform expression of the *gusA* gene (Figure 2). In contrast, the progeny of selfed parental *Arabidopsis* plants did not express detectable GUS activity (data not shown). This observation clearly demonstrated the efficient operation of FLP recombinase in catalyzing excisional DNA recombination, indicating that the FLP/*FRT* recombination system functions in *Arabidopsis* plants.

Expression of antisense bcp1 *gene in* Arabidopsis *causes male sterility*

The *Arabidopsis bcp1* gene is active in both diploid tapetum and haploid microspores. It has been shown to be essential for pollen fertility (Xu *et al.*, 1995a). To induce male sterility in *Arabidopsis*, two constructs, p*bcp1*:a*bcp1* and p35S:*abcp1* (Figure 1c,d), containing antisense *bcp1* gene, were introduced separately into *Arabidopsis thaliana* ecotype Columbia using *A. tumefaciens*-mediated transformation. Transgenic plants were screened from two independent transformation events by spraying with Basta. Of 117 primary transgenic plants (64 from p*bcp1*:a*bcp1* transformation and 53 from p35S:a*bcp1* transformation), four from the p*bcp1*:a*bcp1* and two from the p35S:a*bcp1* transformants were completely male sterile, without the formation of normally elongated siliques and seeds as observed in the wild-type plant

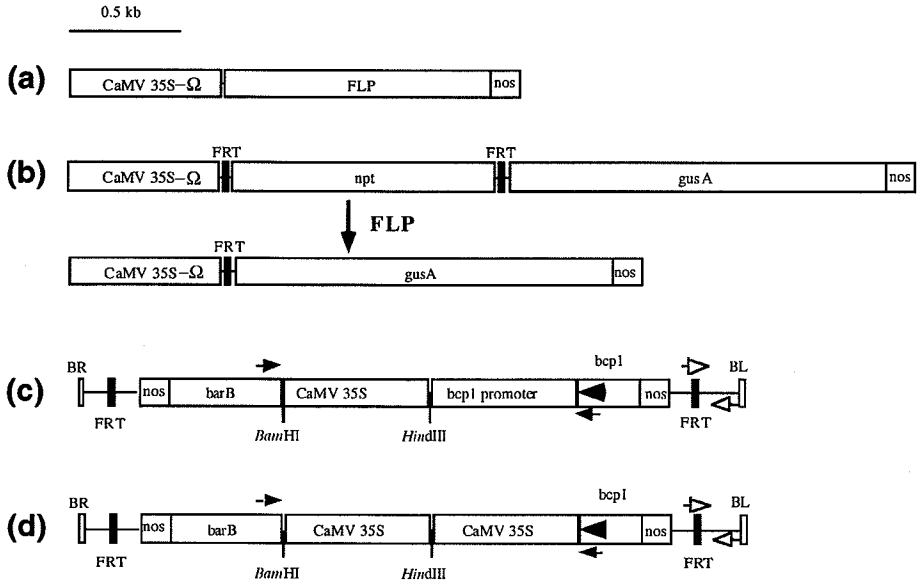


Figure 1. Binary-vector constructs used for *Arabidopsis* transformation.
(a) FLP expression vector pJFLO. The yeast FLP recombinase gene is under the control of CaMV 35S promoter and the omega translational enhancer sequences of TMV (Sleat *et al.*, 1987). Only the FLP cassette within the binary vector is shown here.
(b) *FRT* recombination–reporter construct pFFG (only the *FRT* cassette within the binary vector was shown). The *neomycin phosphotransferase* (*npt*) gene flanked by directly oriented *FRT* sites serves to separate the enhanced CaMV 35S promoter and the *gusA* coding region. Excision of the blocking sequence (*npt* gene) by FLP brings together the 35S promoter and the downstream *gusA* reporter gene, giving rise to *gusA* expression.
(c,d) Constructs with antisense *bcp1*, p*bcp1*:a*bcp1* and p35S:a*bcp1*, were used to transform *Arabidopsis* to generate male-sterile plants. Small filled and open arrows in each construct indicate the position of two pairs of primers used for PCR amplification. Large blocked arrows show the orientation of the *bcp1* coding region.

(Figure 3a). All the six male-sterile plants were phenotypically indistinguishable.

PCR assays on genomic DNA extracted from leaves were performed on all six male-sterile plants. Transgenes were identified in all the transformants (Figure 4). Southern analysis using the *bar* gene as probe revealed one transgene insertion in four of the six male-sterile plants, whereas multiple insertions of the transgenes were identified in the other two male-sterile plants (Figure 5a).

Removal of antisense bcp1 *gene restores fertility and produces hybrid plants*

To determine whether the FLP recombinase can efficiently function to remove the antisense *bcp1* gene between two *FRT* sites and restore plant fertility, all six hemizygous male-sterile transformants (T_0) were cross-pollinated with pollen from FLP-expressing plants (homozygous). If FLP does not function to excise antisense *bcp1* gene, there should still be a 1 : 1 ratio of fertile to sterile plants, i.e. one-half of the gametes would not contain the antisense gene provided that the transgenic plants have a single-locus integration event. On the contrary, if expression of FLP in the hybrid leads to the removal of the antisense *bcp1* gene, then all the plants in the progeny should be fertile.

Indeed, all progeny from the crosses of FLP-expressing plant to the six hemizygous male-sterile plants produced normal siliques and seeds (Figure 3b). To identify plants with transgene genotypes, we carried out PCR on the genomic DNA from hybrid progeny using primers specific to part of the *FRT* site and the sequence close to the left border (BL) of T-DNA (Figure 1c,d). First, we ran PCR on the genomic DNA of 120 randomly chosen hybrid progenies from the crosses of an FLP-expressing plant to the four male-sterile lines containing a single transgene insertion. Approximately half (57) gave amplification of the expected 0.3 kb fragment (Figure 6), indicating that 50% of the hybrid plants had the transgene genotypes. More PCR reactions were then carried out on the genomic DNA of hybrid plants from crosses of the FLP-expressing plant to the six male-sterile lines until 60–90 individuals with transgene genotypes were identified in each of the six crosses for further analyses. The fact that all hybrid plants with transgene genotypes grew normally suggests that the excisional recombination catalyzed by FLP in hybrid progenies should have selectively removed the antisense *bcp1* gene and *bar* gene located between two *FRT* sites, resulting in restoration of fertility.

Genomic analysis of the hybrid progenies with transgene genotypes from all six crosses showed removal of

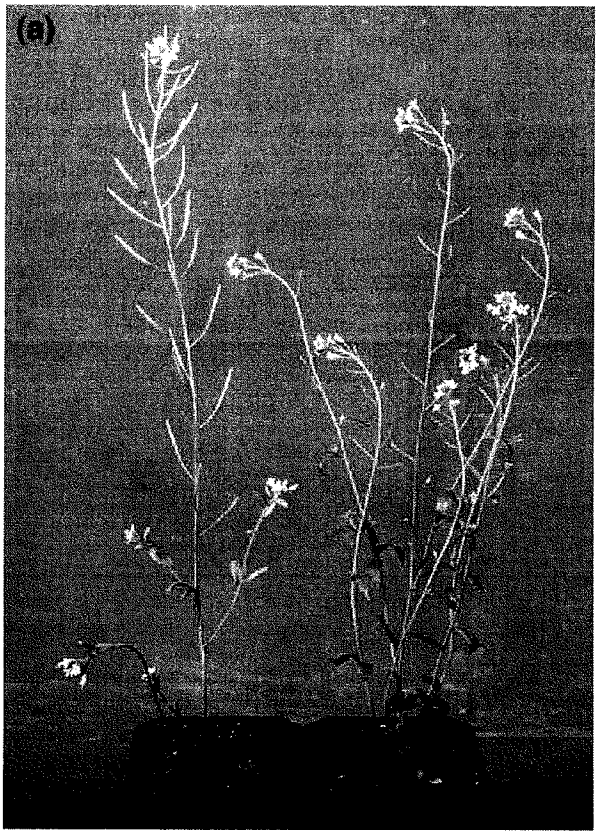
the inserted antisense *bcp1* gene (Figures 4 and 5a,b), confirming that the expression of the antisense *bcp1* gene caused the male sterility, and that its removal restored fertility. Thus the crossing event that brought FLP to its target *FRT* sites in male-sterile plants led to the production of fertile hybrid plants. It should be noted that 2% of the hybrid progenies (two out of 90) derived from one male-sterile p35S:a*bcp1* transformant (M_1) showed a very faint band when their genomic DNA was probed with *bar* gene (Figure 5a, lane T_1–M_1). However, the pollen development in these hybrid plants did not appear to be affected by the presence of this small amount of antisense *bcp1* gene product: all the plants are fertile and grow normally.

Figure 2. Example of histochemical staining of GUS activity in transformed *Arabidopsis* hybrid seedling obtained from cross-pollination between FLP-expressing and *FRT*-containing plants.

Discussion

The present study has demonstrated that FLP recombinase of yeast expressed in transgenic *Arabidopsis* can function in excisional DNA recombination. The expression of antisense *bcp1*, driven by its own promoter or by a constitutive 35S promoter, causes male sterility in *Arabidopsis*. Cross-pollination with pollen from a FLP-expressing plant brings FLP recombinase into the male-sterile plant, and the function of FLP results in the removal of the antisense *bcp1* cassette, completely restoring plant fertility in the hybrid progeny. Thus the FLP/*FRT* system is suitable for plant hybrid production.

In recent years a series of genes has been identified that are involved in normal pollen development in many plant species, including maize (Hanson *et al.*, 1989), rice (Xu *et al.*, 1995b; Zou *et al.*, 1994), tomato (Twell *et al.*, 1989), *Brassica campestris* (Theerakulpisut *et al.*, 1991), and *Arabidopsis* (Xu *et al.*, 1995a). The perturbation of their function leads to male-sterile phenotypes (Moffatt and Somerville, 1988; Xu *et al.*, 1995a). Foreign cytotoxic genes that specifically block pollen development can also be used to produce male sterility (Mariani *et al.*, 1990; Tsuchiya *et al.*, 1995). The male sterility caused by either of these systems is expected to be restored to fertility by specific gene deletion with the FLP/



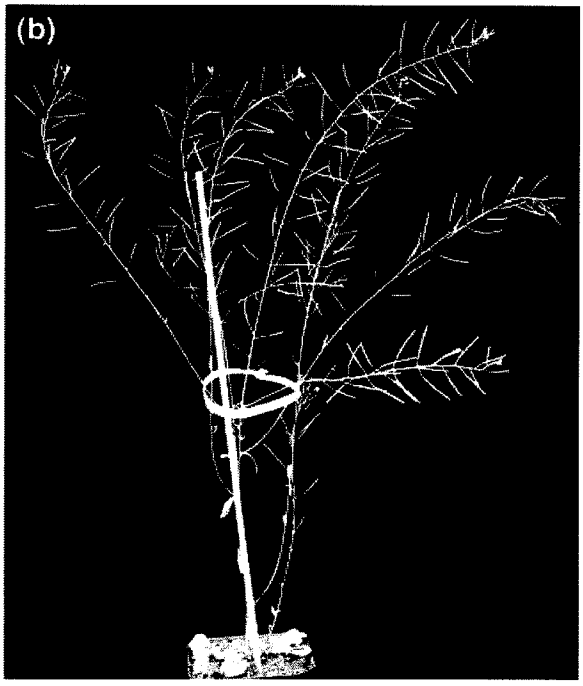


Figure 3. Male-sterile phenotype of transgenenic *Arabidopsis thaliana* plants carrying *bcp1* promoter-antisense transgene.
(a) Example of siliques from wild-type plant (left) and the transgenic male-sterile plant M9 (right). Note the shortened siliques in the male-sterile plants.
(b) Fertile T_1 hybrid plants obtained after excison of the antisense *bcp1* gene.

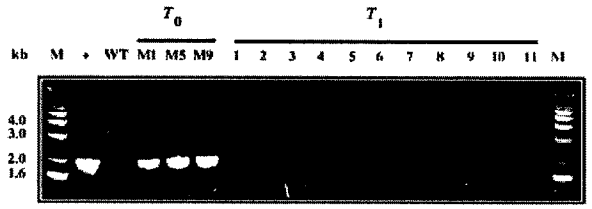


Figure 4. Ethidium bromide-stained agarose gel showing PCR products amplified from the genomic DNA of three male sterile T_0 *Arabidopsis thaliana* plants (M_1, M_5 and M_9) and 11 randomly chosen fertile hybrid T_1 plants with transgene genotypes.
Primers designed for PCR amplification of a DNA fragment between *bcp1* and *bar* genes were as described in Experimental procedures and shown in Figure 1(c,d). Negative control, wild-type plant (WT); positive DNA control, PCR product of the p*bcp1*:a*bcp1* construct (+). A molecular weight ladder (M) is shown.

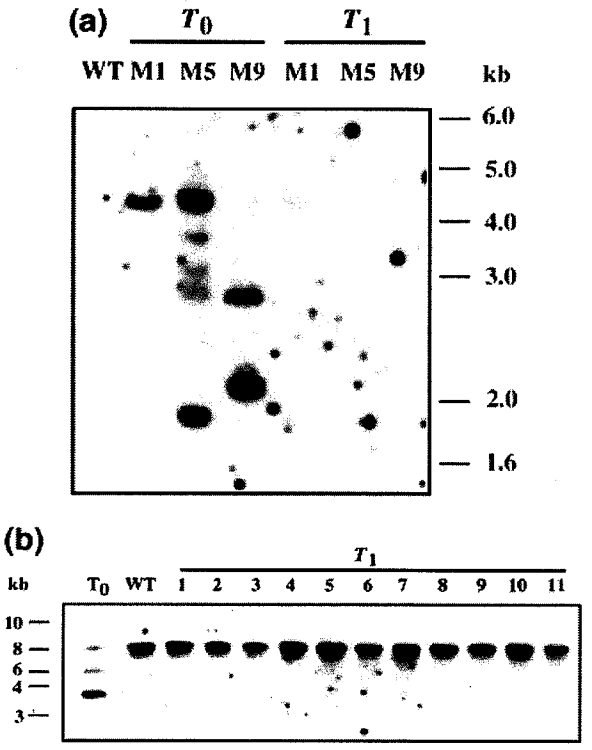


Figure 5. Southern blots of DNA and *bcp1* antisense gene.
(a) Southern blot of DNA of three male-sterile T_0 plants and fertile hybrid T_1 progeny plants from each of the T_0 male-sterile plants. Genomic DNA was digested with *Bam*HI and the *bar* gene was used as the probe. T_1 progeny plants with transgene genotypes correspond to T_0 parents as labeled.
(b) Example of Southern blot of *bcp1* antisense gene. The genomic DNA of male-sterile T_0 plant M9 and its 11 fertile hybrid T_1 progeny plants was digested with *Hin*dIII; *bcp1* was used as the probe. The antisense in T_0 was present as the two lower molecular-weight bands. The endogenous *bcp1* was revealed in T_0 as well as all T_1 progeny with transgene genotypes.

FRT recombination system. This provides a much more flexible method for the production of hybrid plants by genetic engineering strategies.

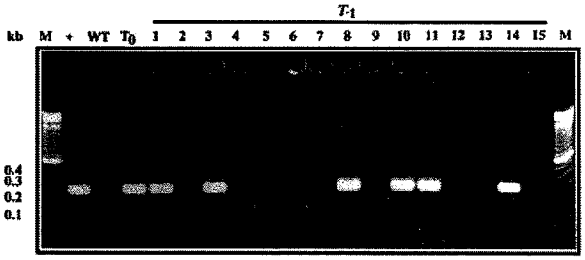


Figure 6. Ethidium bromide-stained agarose gel showing PCR products amplified from the genomic DNA of one male-sterile T_0 *Arabidopsis thaliana* plant (M_1) and its 15 fertile hybrid T_1 plants with transgene genotypes.
Primers designed for PCR amplification of a DNA fragment between the second *FRT* site and the left border (BL) of T-DNA were as described in Experimental procedures and shown in Figure 1(c,d). Negative control, wild-type plant (WT); positive DNA control, PCR product of p35S:a*bcp1* construct (+). A molecular weight ladder (M) is shown.

Our results also indicate that both a constitutive and a tissue-specific promoter can drive antisense gene expression and consequently result in male sterility. This implies that an inducible promoter system could be used for driving expression of antisense or cytotoxic foreign genes at will. Thus the genes controlling male sterility could be kept silent in transgenic plants during seed multiplication, and then turned on to produce male-sterile plants for use in hybrid seed production. Such regulation of the expression of foreign genes in plants has been achieved with promoters responsive to environmental stimuli (Ainley and Key, 1990; Kyozuka *et al.*, 1993; Lyznik *et al.*, 1995) or synthetic chemicals (Ayoma and Chua, 1997; Caddick *et al.*, 1998; Gatz, 1997; Mett *et al.*, 1993; Ward *et al.*, 1993).

FLP recombinase has been successfully introduced into tobacco and shown to function efficiently in the progeny of crosses made between primary transformed tobacco plants (Kilby *et al.*, 1995; Lloyd and Davis, 1994). The results presented here demonstrate that in *Arabidopsis*, FLP recombinase can also be constitutively expressed and can catalyze DNA recombination efficiently. Previous efforts to obtain transgenic *Arabidopsis* plants able to constitutively express functional FLP recombinase were unsuccessful. FLP recombinase showed either no activity in plants (Lloyd and Davis, 1994) or poor activity (Sonti *et al.*, 1995), or functioned only when using an inducible heat-shock promoter (Kilby *et al.*, 1995). This difference might relate to the instability of the FLP recombinase expressed in transgenic *Arabidopsis* due to gene silencing or effects of the positions of the transgenes inserted in the host genome (Matzke and Matzke, 1998). The structure of the FLP gene itself may also play an important role. In our studies we used the FLP gene possessing the consensus sequence for plants around the ATG translation initiation codon, i.e. AACA*ATG* (Joshi, 1987; Lütcke *et al.*, 1987). This is in contrast to the consensus sequence found in animals, i.e. CACC*ATG*, which is also the case for the original *FLP*

gene (Hartley and Donelson, 1980). The use of the *FLP* gene with the plant consensus sequence around the ATG codon may have an exceptionally positive impact on the correct expression of FLP in transgenic *Arabidopsis*, resulting in its high efficiency in catalyzing DNA recombination.

In the present study we also observed that about 2% (two out of 90 analyzed) of the hybrid progenies derived from one male-sterile p35S:a*bcp1* transformant (M_1) showed a very faint band when their genomic DNA was probed with the *bar* gene (Figure 5a, lane T_1–M_1), although pollen development in these hybrid plants did not appear to be affected by the presence of this small amount of antisense *bcp1* gene product, and the plants are fertile and grow normally. This suggests that in some of the hybrid plants derived from this male-sterile line, the FLP excision of the foreign gene between two *FRT* sites is not complete in all cells. One possible explanation could be that DNA methylation in the *FRT* sites hinders the binding of the FLP protein, resulting in the failure of DNA excision. Environmental factors could also be responsible for the failure of DNA excision. Another possibility could be that, in some cells, the excised antisense *bcp1* gene cassette occasionally integrated again into the host genome, but at a different location than its original site. In any case, further work is needed to determine why a small portion of the hybrid plants derived from one particular male-sterile line do not have complete FLP-mediated DNA excision, whereas excision was complete in all the hybrid plants derived from the other five male-sterile lines.

In conclusion, we have shown the constitutive expression of site-specific recombinase FLP in *Arabidopsis*. Moreover, the expressed FLP in transgenic *Arabidopsis* was completely functional and capable of excising a male sterility-causing antisense gene when crossed with a male-sterile plant, producing fertile hybrid seeds. While this system was tested in *Arabidopsis*, it remains to be seen how site-specific recombinase will perform in agriculturally important crop plants. We have already introduced the site-specific recombination systems into rice, and the preliminary results are very promising. The application of the FLP/*FRT* system in producing hybrid rice is currently under way. We believe that the site-specific recombination systems can be successfully applied in crop plants as a useful alternative method for hybrid seed production.

Experimental procedures

Isolation of bcp1 *gene and its 5′ region*

The *Arabidopsis bcp1* gene was cloned by PCR amplification from the ecotype Columbia genomic DNA. Primer (5′-GTCGTCGTGGTTGCCCTCG-3′) specific to the 5′ region and primer (5′-CGACGACCGCAGAGACGCC-3′) specific to the 3′ region of the *bcp1* coding sequence were used for the amplification of a 0.3 kb fragment, which was then cloned into the PCR-Script SK(+) vector. Nucleotide sequence of the PCR-amplified fragment corresponded to the sequence data published by Xu *et al.* (1995a) for the *bcp1* gene isolated from *A. thaliana* ecotype Landsberg erecta. A 0.8 kb region of the 5′ regulatory region of the *bcp1* gene was isolated by screening an *A. thaliana* ecotype Columbia genomic library with the PCR-amplified *bcp1* gene as probe.

Plasmid constructions

Plasmids pJFLO (*FLP* gene expression construct) and pFFG (*FRT* recombination–reporter construct), illustrated in Figure 1(a,b) were essentially as described by Bar *et al.* (1996) with the following modifications: the FLP coding region was amplified by PCR from pOG44 (Stratagene, La Jolla, CA, USA) using the primers 5′-CTGCAGCCCAGTCGACAACAATGCCACAATTTG-3′ and 5′-TTATGCTTAAATCCCGGGTTATATGCGTCT-3′, specific to the 5′ and 3′ ends of the FLP-coding region, respectively. These primers were designed to direct a plant optimal translational modification sequence AACA immediately adjacent upstream of the ATG initiation codon, as well as creating *Sal*I and *Sma*I sites at the 5′ and 3′ ends of the amplified FLP product (termed FLPO), enabling its insertional replacement of FLP in pJFL (Bar *et al.*, 1996), generating pJFLO. Two antisense constructs, p35S:a*bcp1* and p*bcp1*:a*bcp1* (Figure 1c,d), were prepared as follows: the PCR-amplified *bcp1* gene was released as a *Pst*I–*Sac*I fragment and fused in the reversed orientation with the CaMV35S promoter to replace the *gusA* coding sequence in pFFG (Bar *et al.*, 1996). The resulting 35S promoter–antisense *bcp1* fusion fragment was released and ligated into the *Hin*dIII and *Eco*RI sites of the binary vector pSB11 (Komari *et al.*, 1996). One *FRT* sequence was then inserted into the *Eco*RI site, while another *FRT* site together with a selectable marker *bar* gene (under the control of 35S promoter) was ligated into the *Hin*dIII site of this plasmid, giving rise to the p35S:a*bcp1*. To obtain p*bcp1*:a*bcp1*, the pSB11 binary vector, containing the 35S promoter–antisense *bcp1* fusion fragment and one *FRT* site, was cut with *Hin*dIII and *Sac*I to release the 35S promoter. The *bcp1* promoter was then inserted into the *Hin*dIII and *Sac*I sites followed by the ligation of the second *FRT* site as well as 35S promoter-driven *bar* gene into the *Hin*dIII site. The correct orientation of the gene in the plasmid constructs was confirmed either by sequencing or by restriction analyses. All the expression constructs were mobilized into *Agrobacterium tumefaciens strain* LBA4404 by conjugation with a helper plasmid pRK2013 (Koncz and Schell, 1986).

Plant maintenance and transformation

Arabidopsis thaliana ecotype Columbia plants were used for transformation experiments. Seeds were sown into plastic pots containing MetroMix 360 soil mix. After 2 days incubation at 4°C, plants were grown in growth chambers at 24°C, 16 h light/8 h dark photoperiod, and with 60–80% relative humidity. Plants were transformed by vacuum infiltration essentially according to Bechtold *et al.* (1993). Transgenic plants were selected on medium containing kanamycin sulfate (50 μg ml^{-1}) or by spraying a 0.5% of Basta solution on the T_0 transformants. Green plants surviving the antibiotic or herbicide treatment were retained for further analyses.

Cross-pollination

Flowers that contained pistils covered by sepals and petals were hand-pollinated when the stamens were about half as high as the

pistils and anthers were green and not shedding pollen. The FLP-transformed plants were used as a source of pollen that was collected with cotton buds from open flowers. The cross-pollinated flowers were marked and all lower siliques on the same plant were removed. After an additional 3 weeks the seeds were collected, dried, and then planted for the production of hybrid progeny seedlings.

Staining for GUS activity

GUS activity in hybrid progeny seedlings obtained from cross-pollination was assayed by histochemical staining with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc, Biosynth AG, Staad, Switzerland) as described by Jefferson (1987). Whole seedlings were incubated at 37°C overnight in 100 µl reaction buffer containing X-gluc. Prior to photography seedlings were destained in 70% ethanol.

DNA extraction and analysis

Genomic DNA was extracted from about 200–500 mg fresh leaves, essentially as previously described (Luo *et al.*, 1995). DNA (5 µg) was digested with *Hin*dIII or *Bam*HI according to the supplier's instructions (Biolabs). Fragments were size-separated through a 0.8% (w/v) agarose gel and blotted onto a Hybond-N^+ membrane (Amersham). The DNA fragment used as a probe was radiolabeled by random priming with a kit from Amersham, and the Southern blots were processed as described by Sambrook *et al.* (1989).

Polymerase chain reaction

The two primers designed to amplify a DNA fragment between *bcp1* and *bar* genes in the antisense *bcp1* construct were as follows: 5′-GTCGTCGTGGTTGCCCTCG-3′ corresponding to the 5′ end of the *bcp1* gene and 5′-CGGCGGATGTCGGCCGGG-3′ corresponding to the 5′ end of the *bar* coding region (Figure 1c,d). Another pair of primers designed to amplify a DNA fragment between the second *FRT* site and the left border (BL) of T-DNA were as follows: 5′-GGAACTTCGGAATGATCTCC-3′ corresponding to part of the *FRT* site and its adjacent region, and 5′-GATGAAGTGACAGATAGCTGG-3′ corresponding to the sequence close to the left border (BL) of T-DNA (Figure 1c,d). The reaction mixtures (25 µl total volume), overlaid with a drop of mineral oil, consisted of 50 mM KCl, 10 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% (w/v) Triton X-100, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.5 µM of each primer, 0.2 µg of template DNA, and 1 unit of Taq DNA polymerase (Qiagen). Amplification was performed in a Perkin-Elmer Cetus thermal cycler programmed for 25 cycles of 1 min at 94°C (denaturation); 2 min at 55°C (hybridization); 3 min at 72°C (elongation); and a final elongation step at 72°C for 10 min. PCR products were separated in a 0.8% or 1.5% (w/v) agarose gel and detected by staining with ethidium bromide.

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Chemical-regulated, site-specific DNA excision in transgenic plants

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We have developed a chemical-inducible, site-specific DNA excision system in transgenic *Arabidopsis* plants mediated by the Cre/*loxP* DNA recombination system. Expression of the Cre recombinase was tightly controlled by an estrogen receptor-based fusion transactivator XVE. Upon induction by β-estradiol, sequences encoding the selectable marker, Cre, and XVE sandwiched by two *loxP* sites were excised from the *Arabidopsis* genome, leading to activation of the downstream *GFP* (green fluorescent protein) reporter gene. Genetic and molecular analyses indicated that the system is tightly controlled, showing high-efficiency inducible DNA excision in all 19 transgenic events tested with either single or multiple T-DNA insertions. The system provides a highly reliable method to generate marker-free transgenic plants after transformation through either organogenesis or somatic embryogenesis.

Keywords: DNA excision, Cre/*loxP*, marker-free transformants, transgenic plants, XVE

The use of transgenic technology for agricultural purposes has encountered several challenges. One concern is related to the presence in transgenic crop plants of marker genes conferring antibiotic or herbicide resistance. Although no scientific basis has been determined for these concerns, removal of marker genes would likely hasten the public acceptance of transgenic crops.

To this end, several systems for the generation of marker-free transgenic plants have been described. One rather time-consuming approach is to excise or segregate a marker gene from a target gene by sexual crosses or retransformation[1-9]. A second approach is based on overexpression of the isopentenyl transferase (*ipt*) gene[10,11]. In an early attempt, Ebinuma and coworkers developed an *ipt*-based transformation system with low efficiencies (0.5–1.0%)[12]. Recently, these researchers reported an improved marker-excision system using the R recombinase controlled by an inducible promoter, and obtained five marker-free plants from 37 *ipt*-shooty lines[13]. However, the lack of genetic data makes it uncertain whether DNA recombination had occurred in the germlines of these plants. Moreover, this system is not suitable for most economically important crops, which are regenerated through somatic embryogenesis rather than cytokinin-dependent organogenesis. In an independent effort, Kunkel *et al.*[14] placed *ipt* under the control of the GAL4-VP16-glucocorticoid receptor (GVG)-inducible expression system[15] enabling inducible *ipt* expression and regeneration of transgenic plants. Although this method improved the transformation efficiency, the non-plant 35S-*GVG* and *UAS-ipt* transgenes may also raise public concerns. Additionally, the GVG-*ipt* system can only be used for the limited number of plant species that depend on organogenesis for regeneration.

To develop a reliable system for excision of selectable markers, it is important to distinguish successful DNA recombination events in germline cells from those in somatic cells. Whereas marker excision from somatic cells may be useful for vegetative propagation of transgenic plants[12,13,16], the technology cannot be applied to most crop plants, which are propagated by seeds. To produce marker-free progeny, successful DNA recombination must occur in gametes or their L2 progenitor cells in the shoot apical meristem[17-20]. Therefore, the general utilization of a marker excision system depends largely on the DNA excision efficiency in germline cells.

We present here a chemical-inducible, site-specific DNA excision system in transgenic *Arabidopsis* plants, termed CLX (for Cre/*loxP* DNA excision system), controlled by the XVE system[21]. Compared to previously reported systems, the CLX system is tightly controlled and DNA excision can be induced at high efficiency. More important, this system is useful for all types of explant regeneration either by organogenesis or somatic embryogenesis.

Results

A chemical-inducible Cre/*loxP* DNA recombination system. Our strategy was to transform and regenerate transgenic plants using any conventional selectable marker, and subsequently to remove the marker from the host plant genome by chemically regulated site-specific DNA excision. The XVE-inducible expression system[21] was chosen to construct the CLX system (Fig. 1). The bacteriophage P1 Cre recombinase, which specifically recognizes *loxP* sites both *in vivo*[22] and in plant cells[2,23], was placed under the control of the XVE system. Because the O^{LexA}-46 promoter has background expression in bacterial cells, the Cre coding sequence was interrupted by a short intron to prevent bacterial expression of *cre*. The *cre-int* fusion gene was generated by inserting intron 5 of the *Arabidopsis KOR*1 gene[24] between codons 144 and 145 of the *cre* sequence. Nucleotides 4–6 of the intron (TTG) were mutated to AGT to better match the splicing consensus in *Arabidopsis*. A kanamycin-selectable marker was placed between the *XVE* and the *cre* transcription units. These three transcription units were flanked by two *loxP* sites so that β-estradiol-induced DNA recombination would remove all these components, leading to the activation of the downstream *GFP* gene by the G10-90 promoter[25].

The CLX vector pX6-GFP was introduced into *Arabidopsis* by *Agrobacterium*-mediated root culture transformation[26]. Forty-three putative transformed shoots (T_0) were generated after four to five weeks of culturing on a kanamycin-containing shoot regeneration medium. Five shoots were transferred onto an inductive medium, and the remaining 38 shoots were transferred onto

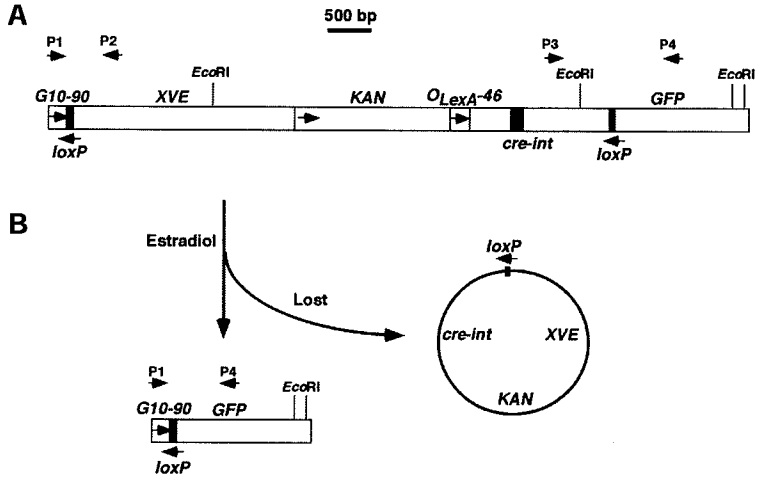

Figure 1. A schematic diagram of the CLX vector and β-estradiol-induced DNA excision. (A) Structural features of the CLX vector pX6-GFP. (See ref. 21 for details of the XVE system.) Three transcription units are located within the two *loxP* sites: *XVE* consists of the coding sequence of the XVE hybrid transactivator terminated by the *rbcs* E9 polyA addition sequence, and is activated by the *G10-90* promoter upstream of the *loxP* site; *KAN* consists of the nopaline synthase (*NOS*) gene promoter, the coding sequence of the neomycin transferase II (NPTII), and the *NOS* polyadenylation sequence; *cre-int* consists of eight copies of the *LexA* operator sequence fused to the −46 CaMV*35S* promoter, the coding sequence of Cre interrupted by an intron and terminated by the *NOS* polyadenylation sequence. Downstream of the second *loxP* site, the *GFP* cDNA was terminated by the *rbcs* 3A polyA addition sequence. Arrows inside squares indicate the direction of transcription. P1 through P4 denote primers used for PCR analysis shown in Figure 2A. Four *Eco*RI sites (used for genomic Southern blot analysis in Fig. 2B) are located at nucleotides 1,796, 5,615, 7,326, and 7,496, respectively. (B) Putative products of the β-estradiol-induced site-specific DNA recombination.

Murashige–Skoog (MS) medium[27]. After induction for two weeks, uniform GFP fluorescence was detected in roots of all five explants cultured on the inductive medium, whereas no *GFP* expression was observed in explants grown on the noninductive MS medium (15 lines examined). All 43 independent lines were then transferred to soil, and T_1 seeds were obtained from 23 lines including 3 lines (numbers 1–3) previously treated with β-estradiol at the T_0 generation. The death before flowering of the remaining 20 lines was presumably due to poor root growth and development.

Genetic analysis of putative T_0 recombinants. T_1 seeds from transgenic lines 1, 2, and 3, treated with β-estradiol in the T_0 generation, were germinated on MS medium and analyzed for *GFP* expression. The proportion of GFP-positive (GFP^+) progeny was less than that expected for a dominant gene (column 2, Table 1). To estimate the recombination frequency, the transgenic locus number was first determined by transferring all GFP-negative (GFP^-) progeny either to the inductive or the selective medium, and additional T_1 progeny showing either β-estradiol-induced *GFP* expression or kanamycin resistance (KAN^R) were identified. It appeared that lines 1 and 3 contained a single transgenic locus, and line 2 contained possibly two transgenic loci (column 3, Table 1). From the number of originally GFP^+ progeny and the transgenic locus numbers, we estimated that DNA recombination had occurred in ~29–66% of germ cells.

In line 2, whereas both transgenic loci appeared to undergo successful DNA recombination upon induction, excision at neither locus was complete. Formation of genetic chimeras in these T_0 transgenic plants could be due to inaccessibility of the L2 progenitor cells to the inducer and inducer instability as well as positional effects of the T-DNA insertions (see below). In lines 1 and 3, T_2 progeny of the putative T_1 recombinants, expressing *GFP* without the inducer, showed a complete loss of the KAN^R marker gene and a mendelian segregation for *GFP* expression in all 17 families tested (Table 1), suggesting complete and precise DNA recombination.

DNA recombination is highly inducible in all tested transgenic lines. To investigate whether the β-estradiol-induced DNA excision occurred in each of the transgenic lines, T_1 seeds from the 20 noninduced transgenic lines (numbers 4–23) were germinated on either the selective or the inductive medium. Four independent lines (numbers 9, 13, 18, and19) appeared to be nontransgenic escapes based on genetic and molecular analyses. No *GFP* expression was detected in the remaining 16 transgenic lines when grown on the selective medium, but all lines showed individuals with uniform *GFP* expression after β-estradiol treatment, indicating that the CLX system in all of these lines was tightly controlled and highly responsive to the inducer. Within each T_1 line, GFP^- plants were kanamycin-sensitive (KAN^S) after transfer to the selective medium, indicating that they were wild-type progeny. Based on the segregation patterns of both the selection marker and *GFP* expression, four lines (numbers 6, 8, 11, and 22) appeared to contain a single transgenic locus whereas six lines (numbers 4, 5, 7, 12, 14, and 16) apparently contain multiple transgenic loci. In these 10 transgenic lines, both the selection marker and the β-estradiol-induced *GFP* expression segregated in a mendelian manner. The transgene copy number was inherently difficult to determine in lines

Table 1. Genetic analysis of T_1 and T_2 progeny derived from putative T_0 recombinants

	T_1[a]		T_2 (family)[d]		
Line	GFP^+/GFP^- (%)[b]	Segregation of transgene[c]	KAN^R/KAN^S	GFP^+/GFP^- Heterozygous	GFP^+/GFP^- Homozygous
1	18/64 (29.3)	63/19	0/1,274 (8)	941/307 (7)	90/0 (1)
2	33/20 (66.4)	53/0	Variable[e]	Variable[e]	Variable[e]
3	18/26 (54.5)	32/12	0/1,235 (9)	652/211 (6)	240/0 (3)

[a]Grown on MS medium without kanamycin or β-estradiol. *GFP* expression was examined three to seven days after germination.
[b]Excision efficiency (%): the ratio of observed to projected (based on a 3:1 or 15:1 segregation) GFP^+ progeny.
[c]GFP^- plants were transferred onto a kanamycin-selective or an inductive medium. Additional T_1 progeny that showed KAN^R or *GFP* expression were identified. Therefore, the segregation indicates the ratio of the sum of seedlings showing KAN^R and GFP^+ to these without either trait.
[d]Randomly selected T_1 GFP^+ seedlings were transferred to soil. Seeds were collected from individual T_1 plants and placed on different media. The numbers of tested T_2 families are given in parentheses. KAN^R / KAN^S plants were germinated on the kanamycin-selective medium. KAN^R or KAN^S phenotypes were scored 7–10 days after germination. GFP^+/GFP^- plants were germinated on MS medium only. Heterozygous or homozygous indicates families showing segregation or no segregation, respectively, for *GFP* expression; therefore, the respective T_1 progenitors were most likely heterozygous or homozygous, respectively, for the transgenic locus. When germinated on the inductive medium, a similar segregation pattern was observed for these families.
[e]Both the kanamycin-selective marker and *GFP* expression showed a variety of segregation patterns including 15:1 (two families), 3:1 (one family), and no segregation (five families).

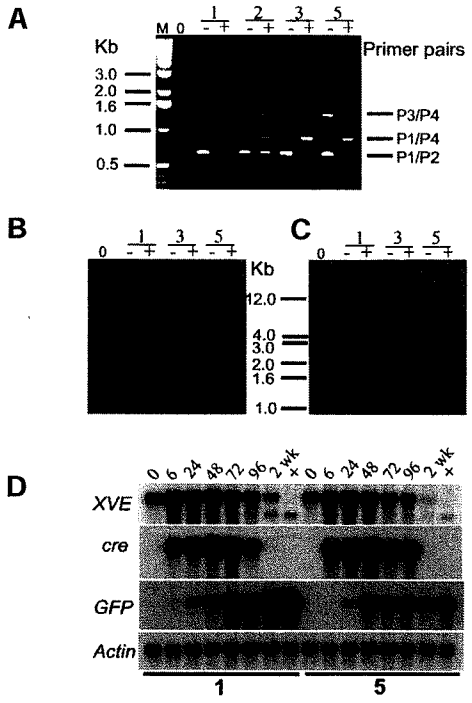

Figure 2. Molecular characterization of β-estradiol-induced site-specific DNA excision in transgenic *Arabidopsis* plants. (A) PCR analysis of genomic DNA prepared from wild-type (lane 0) and transgenic plants (all T_2 plants; lines 1, 2, 3, and 5 as indicated on the top) using primers P1 through P4. The expected PCR products from different combinations of primer pairs are indicated on the left. Uninduced transgenic plants (– lanes) were grown on the selective medium. Putative recombinants (+ lanes) were T_2 progeny (grown on MS medium) derived from β-estradiol-treated T_0 (lines 1, 2, and 3) or T_1 (line 5) transgenic plants. M, DNA molecular weight markers. (B, C) Genomic Southern blot analysis. Blots containing digested genomic DNA (3 μg) from wild-type and transgenic plants were hybridized with either a *GFP* probe (B) or a *NPTII* (C) probe. (D) Northern blot analysis of transgene expression. Two-week-old T_2 plants (lines 1 and 5 as indicated at the bottom) germinated on the selective medium were transferred to the inductive medium and incubated for various time periods (numbered lanes indicate number of hours; 2 wk denotes transgenic plants that were grown on the inductive medium for two weeks; + lanes, same as in part A). Variations in transgene expression in line 5 were presumably caused by the segregation of two transgenic loci among T_2 progeny. Note that the minor band shown in the *XVE* blot is due to residual signal from the *GFP* probe.

10, 15, 17, 20, 21, and 23 because of the small population sizes.

To further characterize the system, we analyzed T_2 progeny from eight transgenic lines (numbers 4, 5, 6, 7, 11, 12, 14, and 16) in detail. The remaining eight lines (numbers 8, 10, 15, 17, 20, 21, 22, and 23), which showed β-estradiol-inducible *GFP* expression in the T_1 generation, were not investigated further. T_2 progeny of the putative T_1 recombinants (treated with β-estradiol and showing *GFP* expression) showed an excision efficiency similar to that of lines 1, 2, and 3. On the other hand, T_1 nonrecombinants showed a characteristic mendelian segregation pattern for both KAN^R and β-estradiol-dependent *GFP* expression (Tables 2 and 3). The above results indicated that the β-estradiol-induced DNA excision occurred in all transgenic lines examined.

Independent DNA recombination in multiple transgenic loci. Segregation analysis suggested that seven transgenic lines (numbers 2, 4, 5, 7, 12, 14, and 16) contained more than one transgenic locus. To address whether all of the transgene copies were excisable upon induction, we selected T_2 families with an approximately 15:1 segregation ratio for KAN^R:KAN^S, which should be heterozygous for both transgenic loci, and tested for the β-estradiol-induced *GFP* expression. If both copies of the transgene could undergo recombination, the *GFP* expression phenotype should also segregate in a 15:1 ratio; or a 3:1 ratio if only one copy underwent recombination. In all seven tested transgenic lines, β-estradiol-induced *GFP* expression showed a segregation pattern similar to that of the KAN^R selective marker (Table 3). Note that the *GFP* reporter gene appeared to uniformly express in the inducer-treated transgenic plants as examined under a fluorescence microscope. Some of these plants, however, were genetic chimeras as shown by subsequent genetic (Table 2 and 3) and molecular analyses (see below). Nevertheless, the above results demonstrated that all T-DNA insertions were fully functional for β-estradiol-induced, independent DNA recombination.

Molecular analyses of β-estradiol-induced DNA recombination. To characterize the β-estradiol-induced DNA recombination at the molecular level, we performed PCR analysis using primers specific for the excised sequences and flanking nonexcised sequences (see Fig. 1). In a nonrecombinant T-DNA, P1/P2 and P3/P4 will amplify two DNA fragments of 653 and 1,376 bp, respectively; P1/P4 will be unable to amplify a 6 kb DNA fragment under the assay conditions. Upon correct DNA recombination, however, P1/P4 will amplify the rejoined *G10-90-loxP-GFP* sequence (990 bp). In uninduced plants, only the P1/P2 and P3/P4 amplified fragments were detected, indicating that the system is tightly controlled. After induction, three DNA fragments were detected in genomic DNA prepared from transgenic line 2 plants containing two transgenic loci. These three amplification products corresponded to the P1/P2 and P3/P4 fragments in nonrecombinant T-DNA and to the P1/P4 fragment in recombinant T-DNA. However, in the remaining three lines tested (single transgenic locus, lines 1 and 3; multiple transgenic loci line 5) only the P1/P4 fragment was detected (Fig. 2A), indicating complete DNA excision. Among other tested lines, three (single transgenic locus lines 6 and 11 and multiple transgenic loci line 14) showed complete DNA excision, and four (transgenic lines 4, 7, 12, and 16; all with multiple transgenic loci) with incomplete DNA excision.

We performed genomic Southern blot analysis to test whether the excised DNA fragment was reinserted elsewhere in the host plant genome. A *GFP* probe detected an *Eco*RI fragment with the expected size in uninduced transgenic plants, whereas larger DNA fragments were detected in recombinant plants (Fig. 2B), presumably representing fusion events between *Arabidopsis* genomic DNA and the remaining *G1090-GFP* transgene (see Fig. 1). Using *XVE*, *cre* (data not shown), and *NPTII* (Fig. 2C) coding sequences as probes, no hybridization signal was detected in recombinant plants, whereas a DNA fragment of the expected size was present in uninduced transgenic plants. This indicates that the excised DNA did not reinsert into the host plant genome. Note that two DNA fragments were detected by the *GFP* probe in line 5, containing two copies of the T-DNA insert, further demonstrating that both copies were indeed functional (Fig. 2B).

Table 2. Genetic analysis of T_2 progeny derived from T_1 nonrecombinants with a single transgenic locus[a]

Line	T_1 Heterozygous (family)		T_1 Homozygous (family)[b]	
	KAN^R/KAN^S	GFP^+/GFP^-	KAN^R/KAN^S	GFP^+/GFP^-
1	203/69 (2)	283/88 (2)	225/0 (2)	161/0 (2)
3	179/67 (2)	278/83 (2)	259/0 (2)	137/0 (2)
6	168/53 (2)	171/56 (2)	179/0 (2)	120/0 (2)
11	250/85 (2)	153/48 (2)	212/0 (2)	120/0 (2)

[a]T_1 plants were grown on the selective medium, and T_2 seeds were used in these experiments. See Table 1 footnotes for other technical details.
[b]No *GFP* expression was detected in any tested KAN^R plants (200 seedlings from eight families).

Table 3. Genetic analysis of T_2 progeny derived from T_1 nonrecombinants with T-DNA insertions in multiple loci[a]

Line	KAN^R/KAN^S (family)	GFP^+/GFP^- (family)
2	248/17 (2)	192/12 (2)
4	90/6 (1)	85/7 (1)
5	256/18 (2)	176/11 (2)
7	113/9 (1)	99/7 (1)
12[b]	115/5 (1)	86/4 (1)
14[b]	123/5 (2)	177/9 (2)
16	263/18 (3)	366/26 (3)

[a]T_1 plants were grown on the selective medium and the resulting T_2 seeds used in these experiments. See Table 1 footnotes for other technical details.
[b]In these two lines, both KAN^R and *GFP* expression appeared to segregate in a ratio greater than 15:1, presumably as a result of linkage between these two transgenic loci.

To monitor the progression of induced DNA recombination, we examined transgene expression by northern blot analysis at different times after β-estradiol treatment (Fig. 2D). The *XVE* fusion gene was expressed in uninduced plants but not in recombinant plants (+ lanes). The *XVE* fusion gene, along with the contiguous excisable DNA segment, was lost upon inducer treatment. As expected, *XVE* expression gradually declined upon extended induction. *cre* expression was tightly controlled by the XVE system without detectable expression in uninduced plants, but strong induction after β-estradiol treatment. Similar to that of *XVE*, *cre* expression gradually decreased with time, presumably as a result of transgene excision and/or inducer instability (ref. 21). Presumably, this instability, as well as the partial accessibility of L2 progenitor cells to β-estradiol, lead to the formation of genetic chimeras in some transgenic lines. In contrast to the declining *XVE* and *cre* expression, *GFP* expression, which is strictly dependent on correct DNA recombination, was detectable after 6 h of induction and progressively increased upon prolonged β-estradiol treatment. Consistent with the above, GFP fluorescence was usually detectable after 12–16 h of β-estradiol treatment.

Discussion

Here we describe a tightly regulated and highly efficient site-specific DNA excision system in transgenic *Arabidopsis* plants. This system has several advantages. First, it is tightly controlled by β-estradiol and, moreover, any leaky expression will result in no regeneration of transgenic plants due to the loss of the selectable marker. Second, site-specific DNA excision can be induced at any given time. Third, upon induced DNA excision, all "used" components of the system including the selectable marker and the XVE system itself, will be removed from the host plant genome. This feature is of utmost importance for the generation of marker-free transgenic plants, and for the generation of transgenic plants carrying multiple transgenes. Fourth, upon induced DNA excision, the target gene is permanently activated, a situation that presents key advantages over transiently induced target gene expression (such as the XVE system itself). If desired, the *G10-90* promoter can also be excised by placing a *loxP* site upstream from the promoter, whereas a promoter of interest can be used to control the target gene. Fifth, because the system can use any conventional selectable marker, it is applicable to any transformation method. Finally, because multiple transgene insertions, either linked or independent, occur frequently during plant transformation, the removal of all copies of the selectable marker from the host plant genome is of concern. The CLX system is capable of efficiently excising DNA sequences from multiple T-DNA insertions, whether unlinked or linked.

Compared to the GST-MAT system, in which 14% of the induced-transgenic lines underwent DNA excision[13], the CLX system appeared to function in all 19 transgenic lines examined. More importantly, we provide compelling genetic evidence showing a high efficiency (29–66%), β-estradiol-dependent DNA recombination in germline cells, which was unknown for the GST-MAT system. With appropriate improvements in the induction conditions, a higher DNA excision efficiency may be expected.

Based on the KAN^S and *GFP* expression phenotypes, each of the 19 tested lines responded to the inducer, and showed uniform somatic *GFP* expression. Nevertheless, genetic and molecular analyses revealed that some of the treated lines were genetic chimeras, presumably caused by incomplete DNA excision in the L2 somatic progenitor cells because of inducer inaccessibility or instability[21]. This problem may be obviated by repeated applications of the inducer, particularly to the shoot meristem region. In any case, this disadvantage of the CLX system should not detract from its use for regulated, DNA excision in transgenic crop plants. As demonstrated here with *Arabidopsis* lines 1, 2, and 3, primary transformants can be immediately treated with the inducer to obtain T_1 progeny with all possible segregation patterns, allowing the recovery of transgenic plants with the desired genotype. The appropriate T_1 plants containing only the target gene can be used immediately for outcross with commercial varieties.

Experimental protocol

Plasmid construction. All molecular manipulations were carried out following standard procedures[28]. Pwo DNA polymerase was used in PCR's according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). To construct pX1, the *PstI/Eco*RI fragment of pMM23 (the Cre coding sequence) and a PCR-generated fragment (the *NOS* poly A addition sequence fused to a *loxP* site; pMM23 primed by CreF/3′-lox2 primers, digested with *Eco*RI) were co-ligated into *PstI/Hin*cII digested pBlueScript vector (Stratagene, La Jolla, CA). A *GFP* cDNA was inserted downstream of the *loxP* site to yield pX-2.

The *cre-int* fusion gene vector pX-3 was constructed by a series of PCR-ligation-PCR reactions. All PCR fragments were treated with T4 DNA kinase before subsequent ligation reactions, which were then directly used for downstream PCR. Appropriate controls with different combinations of PCR primers were included in steps 2 and 3. (1) ligation 1/2: the CreATG/CreQB primed PCR fragment (codons 1–144 of Cre; pX1 as the template) was ligated with a CeInF/CeInB amplified PCR fragment containing intron 5 of the *KOR1* gene[24]. Nucleotides 4–6 of the intron (TTG) were mutated to AGT to better match the splicing consensus in *Arabidopsis*; (2) ligation 3/4: CreATG/CeInB primed ligation 1/2 (Cre 1–144-KOR1 intron 5 fusion fragment) was ligated to CreVF/CreRIB (Val–145 to the end of Cre sequence; pX1 as the template); (3) PCR5: ligation 3/4 was amplified with CreATG/CreRIB primers. The *Eco*RI-digested PCR5 fragment was inserted into *StuI/Eco*RI digested pX2 to yield pX3 consisting of *cre-int-Tnos-loxP-GFP* sequences.

The *G10-90-loxP-XVE* fusion gene (partial XVE sequence) was made by inserting the LoxT1/ERB1359 primed PCR fragment (treated with T4 DNA kinase; pER8 as the template[21]) into the *Sma*I /*Ecl*136II digested pLiG1090 vector[25] to generate pX4. To construct pX5, the *MluI/Xho*I fragment of pER10 (containing 3′portion of the *XVE* transcription unit, the kanamycin transcription unit, and the O^{LexA}-*46* target promoter) and the *SalI/Spe*I fragment of pX3 (containing the *cre-int-Tnos-loxP-GFP* sequences) were co-ligated into the *MluI/Spe*I digested pX4. The CLX vector pX6-GFP was constructed by replacing the *Sse*8337*I/Spe*I fragment of pER8 (ref.21) with that of pX5 (see Fig. 1). Primers used for PCR:

CreF: 5′-CTGGACACAGTGCCCGTGTCGGA-3′
3′-lox2: 5′-GAAGATCTATAACTTCGTATAATGTATGCTATACGAAGT TATGATCTAGTAACATAGATGACACC-3′
CreATG: 5′-CCCGTCGACATGTCCAATTTACTGACCGTA-3′
CreQB: 5′-CTGGTCGAAATCAGTGCGTTCGAA-3′
CeInF: 5′-GTAAGTCTTCTTTTCCTTTACTCTTATCAG-3′
CeInB: 5′-CTGCCAAAATACAGCAAGGCCGAG-3′
CreVF: 5′-GTTCGTTCACTCATGGAAAATAGCGATC-3′
CreRIB: 5′-GCCTTTCCCCGCATGAATAATATTGATG-3′
LoxT1:5′-TAATAACTTCGTATAGCATACATTATACGAAGTTATGAATTAAATCCGGGCGGAATGAAA-3′
ERB1359: 5′- GATGAGGAGGAGCTGGGCCAGCCG-3′

The pX6-GFP sequence has been deposited in the GenBank database

(accession number: AF330636).

Plant materials, growth conditions, and plant transformation. The *Wassilewskija* ecotype of *A. thaliana* was used in all experiments. The selective and inductive media contained MS medium[27] plus kanamycin (50 mg/L) or 17-β-estradiol (2 μM), respectively. GFP fluorescence was examined using a Zeiss Axioskop fluorescent microscope.

PCR analysis and genomic Southern blot analysis. Genomic DNA was prepared with the Plant DNAeasy Prep Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Approximately 50 ng of genomic DNA were used for PCR. The reactions were subjected to 94°C for 20 s, 60°C for 30 s, and 72°C for 2 min for 50 cycles. Primers for PCR analyses:

P1: 5′-CCATCTCCACTGACGTAAGGGAT-3′
P2: 5′-CTCGTCAATTCCAAGGGCATCGGT-3′
P3: identical to CreF.
P4: 5′-TTGTATAGTTCATCCATGCCATG-3′

Genomic Southern blot analysis was carried out following standard methods[28]. Briefly, genomic DNA (3 μg) prepared from wild-type and transgenic plants was digested with *Eco*RI, and separated on a 0.8% agarose gel, and blotted onto a nylon membrane (Stratagene). Blots were hybridized with a *GFP* probe and the other with a *NPTII* probe labeled with the Megaprimer DNA Labeling System (Amersham, Piscataway, NJ).

All other methods, including plant growth and transformation, and RNA manipulations, have been described[21,24].

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RELATED PROCEEDINGS APPENDIX

None.